(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
8 August 2002 (08.08.2002)

PCT

(10) International Publication Number
WO 02/060947 A3

(51) International Patent Classification[7]: C07K 14/705, A61K 38/17

(21) International Application Number: PCT/US02/00509

(22) International Filing Date: 18 January 2002 (18.01.2002)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/265,690 1 February 2001 (01.02.2001) US

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(81) Designated States *(national)*: AE, AG, AL, AM, AT (utility model), AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ (utility model), CZ, DE (utility model), DE, DK (utility model), DK, DM, DZ, EC, EE (utility model), EE, ES, FI (utility model), FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK (utility model), SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZM, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Declarations under Rule 4.17:
— *as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii)) for the following designations AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZM, ZW, ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG)*
— *as to the applicant's entitlement to claim the priority of the earlier application (Rule 4.17(iii)) for the following designations AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZM, ZW, ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG)*

Published:
— *with international search report*
— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments*

(88) Date of publication of the international search report: 31 October 2002

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 02/060947 A3

(54) Title: GLYCOFORMS A FAS LIGAND INHIBITORY PROTEIN ANALOG

(57) Abstract: The present invention provides FLINT analog isoform compositions having specified average sialic acid content per molecule of FLINT analog.

FLINT ANALOG GLYCOFORMS

BACKGROUND OF THE INVENTION

FLINT is a glycoprotein involved in regulating apoptosis. A number of tumor necrosis factor receptor proteins ("TNFR proteins") have been isolated in recent years, having many potent biological effects. Loss of normal activity of these proteins has been implicated in a number of disease states.

Increased activation of the Fas-FasL signal transduction pathway is implicated in a number of pathological conditions, including runaway apoptosis (Kondo *et al.*, Nature Medicine 3(4):409-413 (1997); Galle *et al.*, *J. Exp. Med.* 182:1223-1230 (1995)), and inflammatory disease resulting from neutrophil activation (Miwa *et al.*,. *Nature Medicine 4*:1287 (1998)). "Runaway apoptosis" is a level of apoptosis greater than normal, or apoptosis occurring at an inappropriate time. Pathological conditions caused by runaway apoptosis include, for example, organ failure in the liver, kidneys and pancreas. Inflammatory diseases associated with excessive neutrophil activation include sepsis, ARDS, SIRS and MODS.

One particular TNFR homologue, referred to herein as "FAS Ligand Inhibitory Protein," or "FLINT", binds Fas Ligand (FasL) thereby preventing the interaction of FasL with Fas. FLINT also binds the ligand known as LIGHT to prevent the interaction of LIGHT with receptor LTBR, an otherwise initiating step in a second, independent apoptotic pathway.

Compounds such as FLINT can be used to treat or prevent diseases or conditions in mammals, including humans, that

clinically may correlate with either, or both, of the binding interactions of Fas to FasL and LIGHT to LTBR.

Many eucaryotic secretory proteins including FLINT are modified with one or more oligosaccharide groups (See PCT applications WO0058466, WO0058465, and WO9950413). The state of glycosylation of such proteins can dramatically affect their physical properties and also be important to protein stability, secretion, and subcellular localization. Moreover, proper glycosylation can be essential for biological activity. In fact, some genes from eucaryotic organisms, when expressed in bacteria (e.g., *E. coli*) yield proteins that have little or no activity by virtue of their lack of glycosylation.

Glycosylation occurs at specific locations along the polypeptide backbone and is usually of two types: O-linked oligosaccharides are attached to serine or threonine residues, while N-linked oligosaccharides are attached to asparagine residues when part of the consensus sequence Asn-X-Ser/Thr, where X can be any amino acid except proline. Additionally, the structure and composition of N-linked and O-linked oligosaccharides differ. One type of sugar that is commonly found on N-linked and O-linked oligosaccharides is N-acetylneuraminic acid (hereafter referred to as sialic acid). Sialic acid is usually the terminal residue on both N-linked and O-linked oligosaccharides and, by virtue of its negative charge, may confer acidic properties on a glycoprotein.

FLINT has an N-linked glycosylation site at Asn 173 of SEQ ID NO:3 (Asn 144 of SEQ ID NO:1), and O-linked glycosylation sites at Thr 203 (alternatively, Thr 174 of SEQ ID NO:1); and Thr 245 of SEQ ID NO:3 (alternatively, Thr 216 of SEQ ID NO:1). Under ordinary conditions of

recombinant production, the O-linked site at Thr 216 is substantially less glycosylated than the O-linked site at Thr 174 of SEQ ID NO:1.

Sialic acid content has a direct effect on the PK profile of FLINT analogs. The higher the sialic acid content, the slower the clearance from serum *in vivo*. This observation has heretofore not been disclosed. The present invention relates to the relationship between sialic acid content and *in vivo* clearance of FLINT analogs, and to FLINT analog compositions that have defined level of sialylation. Owing to this relationship, under-sialylated FLINT analog is cleared more rapidly from the serum of primates than FLINT analog with greater sialylation content. Low-sialylated FLINT analog is probably cleared from the circulation upon interaction with certain hepatic receptors, for example, the hepatic asialoglycoprotein binding protein (cf. Morrell et al. J. Biol. Chem. 243, 155 (1968); Briggs, et al. Am. J. Physiol. 227, 1385 (1974); Ashwell et al. Methods Enzymol. 50, 287 (1978)). Therefore, achieving enhanced levels of sialylation is expected to improve the therapeutic utility of FLINT analogs.

It is an object of the present invention to provide isoforms of FLINT analogs having enhanced sialic acid content. The isoforms of the present invention are analogs of FLINT having additional glycosylation sites engineered into the native FLINT sequence. These additional glycosylation sites provide additional sites for sialylation. Pharmaceutical compositions comprising such molecules provide FLINT analog compositions with slower clearance time *in vivo* and enhanced therapeutic benefit.

SUMMARY OF THE INVENTION

The subject invention relates to FLINT analog sialic acid isoforms. Also provided are methods of preparing FLINT analog isoforms and pharmaceutically acceptable compositions comprising same. This invention also relates to therapeutic methods comprising administering a therapeutically effective amount of these FLINT analog compositions to treat and/or prevent diseases or conditions in mammals including humans.

The subject invention relates further to methods of preparing FLINT analog isoforms comprising subjecting material containing FLINT analog to ion exchange chromatography, liquid chromatography, or chromatofocusing, as well as methods to enhance sialylation of FLINT analogs comprising use of enzymatic processes *in vitro*.

DETAILED DESCRIPTION OF THE INVENTION

SEQ ID NO:1 - Mature human FLINT, i.e. native FLINT minus the leader sequence.

SEQ ID NO:2 - Nucleic acid/cDNA encoding mature human FLINT.

SEQ ID NO:3 - Native human FLINT.

SEQ ID NO:4 - Nucleic acid encoding human FLINT.

SEQ ID NO:5 - Oligonucleotide sequence of primer CF119 used in production of analog A12N.

SEQ ID NO:6 - Oligonucleotide sequence of primer CF120 used in production of analog A12N.

SEQ ID NO:7 - Oligonucleotide sequence of primer CF121 used in production of analog A12N.

SEQ ID NO:8 - Oligonucleotide sequence of primer CF122 used in production of analog A12N.

The term "analog" or "FLINT analog" is used herein specifically to mean a FLINT variant having one or more amino acid sequence changes in SEQ ID NO:1 or SEQ ID NO:3, e.g. substitution, addition, deletion, such that one or more additional glycosylation site(s) is present when compared with native FLINT. Said analogs or variants retain the biological activity of FLINT.

As used herein, "average sialic acid content" refers to a quantitative measure of the sialic acid content of a FLINT analog sample preparation expressed as the mole fraction of sialic acid per mole of analog. The term allows comparison of different lots or preparations of FLINT analog. FLINT analog preparations may comprise multiple FLINT isoforms, for example, 0, 1, 2, 3, 4, 5, 6 or more sialic acids per molecule of FLINT analog.

The term "native FLINT" refers to SEQ ID NO:3.

The term "mature FLINT" refers to SEQ ID NO:1.

Description of FLINT analog isoforms provided herein may be referenced against SEQ ID NO:1 or SEQ ID NO:3. Both the native and mature forms of FLINT are within the scope of the invention.

"FLINT analog isoforms" refers to sialic acid variants. Native FLINT can occur with 0, 1, 2, 3, 4, 5, or 6 sialic acid residues per molecule of FLINT. Each additional N-linked glycosylation site would provide 0, 1, 2, 3, or 4 additional sialic acids per molecule of analog; each additional O-linked site would provide 0, 1, or 2 additional sialic acids per molecule of analog. The degree of sialylation will depend on the host cell and growth conditions used in producing recombinant FLINT analogs. Sialylation can be enhanced *in vitro* using an enzymatic process described later in this disclosure.

The term "*N*-glycosylated polypeptide" refers to polypeptides having one or more NXS/T motifs in which the nitrogen atom in the side chain amide of the asparagine is covalently bonded to a glycosyl group. "X" refers to any naturally occurring amino acid residue except proline. The "naturally occurring amino acids" are glycine, alanine, valine, leucine, isoleucine, proline, serine, threonine, cysteine, methionine, lysine, arganine, glutamic acid, asparatic acid, glutamine, asparagine, phenylalanine, histidine, tyrosine and tryptophan. *N*-Glycosylated proteins are optionally *O*-glycosylated.

The term "*O*-glycosyled polypeptide" refers to polypeptides having one or more serines and/or threonine in which the oxygen atom in the side chain is covalently bonded to a glycosyl group. *O*-Glycosylated proteins are optionally *N*-glycosylated.

The nucleotide and amino acid abbreviations used herein are those accepted in the art and by the United States Patent and Trademark Office, as set forth in 37 C.F.R. 1.822 (b)(2).

The present invention relates to sialic acid isoforms of analogs of FLINT in which one or more amino acids of native FLINT are substituted, deleted, or added to create additional glycosylation site(s). Preferred analogs include those having one or two additional N-linked glycosylation sites. N-linked sites are created by introducing an N-linked consensus sequence into the native FLINT sequence. N-linked glycosylation consensus sites comprise the sequence NXS/T.

Contemplated by the present invention are specific analogs of SEQ ID NO:1 having substitutions to create new N-linked glycosylation sites at different positions, including: Ala2 or Ala12 to Asn (1 additional N-linked

site); Pro25, Pro38, Pro126, or Pro171 to Asn (1 additional N-linked site); Arg35 to Asn (1 additional N-linked site); Ser37 to Asn and Pro38 to any amino acid (1 additional N-linked site); Ser166 to Asn (1 additional N-linked site); Leu172 to Asn (1 additional N-linked site); Asp194 to Asn (1 additional N-linked site); Thr114 to Asn and Pro115 to any amino acid (1 additional N-linked site). Also contemplated are substitutions including: Ala12 to Asn and Glu13 to Gln (1 additional N-linked site); Arg34 to Asn and Asp36 to Thr (1 additional N-linked site); Arg35 to Asn, Ser37 to Thr (1 additional N-linked site); Ser132 to Asn, Ser134 to Thr (1 additional N-linked site); Asp194 to Asn, Ser196 to Thr (1 additional N-linked site); Arg35 and Asp194 to Asn (2 additional N-linked sites); Arg34 to Asn, Asp36 to Thr (1 additional N-linked site); Asp194 to Asn, Ser196 to Thr (1 additional N-linked site); Ser132 to Asn (1 additional N-linked site); or Ala12 and Ser132 to Asn and Ser134 to Thr (1 additional N-linked site); Ala12 to Asn, Glu13 to Gln, Asp 194 to Asn, Ser 196 to Thr (2 additional N-linked sites); Arg34 to Asn, Asp36 to Thr, Asp194to Asn, Ser196 to Thr (2 additional N-linked sites).

Glycosylation sites may be introduced into the native FLINT cDNA sequence most conveniently by *in vitro* mutagenesis techniques, well known to the skilled artisan (*See e.g.*, T. Maniatis *et al*. Molecular Cloning: A Laboratory Manual, 2d Ed.(1989), and Ausubel *et al*., eds., Current Protocols in Molecular Biology, Wiley Interscience, New York (1987-1999). For example, synthetic oligonucleotides are designed to incorporate a point mutation at one end of an amplified fragment. Following first strand PCR, the amplified fragments encompassing the mutation are annealed with each other and extended by

mutually primed synthesis. Annealing is followed by a second PCR step utilizing 5′ forward and 3′ reverse end primers in which the entire mutagenized fragment gets amplified and is ready for subcloning into an appropriate vector.

Analogs are generated by site-directed mutagenesis and include additions, deletions, or substitutions of amino acid residues that add sites that are available for glycosylation. Analogs of the invention potentially have a greater number of carbohydrate chains than native FLINT, their sequence having additional N-linked glycosylation sites engineered therein.

FLINT analogs were constructed using the method described in Nelson, R. M. and Long, G. L. (1989), *Anal. Biochem.* 180, 147-151. The method utilizes 4 primers, 2 external (A and D) and 2 internal primers (B and C) that are mutagenic. The strategy involved PCR amplification of a cassette having defined unique restriction enzyme sites surrounding the desired mutation. This cassette was subcloned into a wild-type FLINT vector backbone. Following PCR amplification, inserts were cloned into commercial PCR cloning vector pCR2.1TOPO (Invitrogen) and submitted for DNA sequencing prior to subcloning into an expression vector.

As an example, the cassette for construction of the analog Ala12 to Asn, comprising NheI and KpnI ends, was generated using the following oligonucleotide primers:

CF119 (A): gag cta gcc acc atg agg gcg ctg gag ggg cca ggc ctg tcg ctg

CF120(B): GTC TCG TTG TCC CGC CAT GGG TAG GTG GGT GTT TCT GCC ACT CCG CGT ACA G

CF121(C): ggc aga aac acc cac cta ccc atg gcg gga caa cga gac agg gga gcg gct g

CF122(D): GTC GAT GAC GGC ACG CTC ACA CTC CTC AGC TCC TGG TAC CCT GGT GCT G

By increasing the sialic acid content of the FLINT molecule, analogs of the present invention have an increased clearance time from serum. Analogs having greater sialic acid content than native human FLINT are generated by adding glycosylation sites which do not perturb the secondary or tertiary conformation required for biological activity. Preferably, an analog of FLINT has 1, 2 or 3 additional sites for N-glycosylation. Each additional N-linked site can provide up to four additional sialic acids per molecule of FLINT analog.

According to the present invention, FLINT isoforms can be separated by a variety of techniques including isoelectric focusing (IEF). When placed in a pH gradient and subjected to an electric field, proteins will migrate to the point at which they have no net charge. This is the isoelectric point (pI) of the protein. Each distinct band observed on IEF represents molecules that have a particular pI and therefore the same overall charge, termed an isoform. The term "FLINT isoform" as used herein refers to FLINT preparations having about the same pI, as measured by any suitable technique, and the same amino acid sequence.

Other means for separating FLINT isoforms include fractionation over an ion exchange column. Preferably isoforms are separated by liquid chromatography (LC).

In a preferred embodiment, FLINT analogs are the product of the expression of an exogenous DNA sequence transfected into a non-human eucaryotic host cell (i.e. "recombinant FLINT"). A recombinant FLINT analog is advantageously produced and purified according to the procedures described in commonly owned PCT applications WO 00/58466, WO 00/58465, and WO 99/50413, hereby incorporated by reference. For example, the native FLINT cDNA was incorporated into vector pcDNA3 DHFR which provides the CMV promoter to drive FLINT gene expression and DHFR selection. DG44-C.B4 CHO cells were transfected with linearlized vector by electroporation. For non-selective propagation, cells were grown in Ex-Cell 302 Medium (JRH BioSciences), 1X HT Supplement (GibcoBRL), 1X dextran sulfate (Sigma) and 6 mM L-glutamine (GibcoBRL). For selective growth, cells were placed in Ex-Cell 302 Medium (JRH BioSciences), 1X HT Supplement (GibcoBRL), 1X dextran sulfate (Sigma), 6 mM L-glutamine (GibcoBRL), and Methotrexate (20 mM stock, USP).

After electroporation cells were placed in non-selective growth medium to recover for 72 hours. Plating was done using 96 well dishes. The cells were plated at various cell densities, and under various levels of methotrexate (MTX) selective pressure. When colony formation was visible the plates were screen by ELISA. The wells were moved into 24 well dishes and expanded to generate enough cells for a full expression study evaluation.

Master wells were amplified at different levels of methotrexate. All master wells showed increased levels of expression at the end of the amplification step. Two master wells were cloned using FACS cloning.

Discrete isoforms of a given recombinant FLINT analog are contemplated herein corresponding to FLINT molecules

having from 0, 1, 2, 3, 4, 5, 6, 7, 8 or greater number of sialic acids per molecule of analog. Increasing the number of sialic acid residues per molecule of FLINT has the effect of slowing the clearance of FLINT *in vivo*.

As demonstrated herein, the *in vivo* clearance of FLINT and FLINT analog variants correlates with sialic acid content. (See Examples 3 and 4). Specifically, FLINT analogs having greater sialic acid content, as expressed, for example, by the average sialic acid content, are cleared more slowly than the corresponding analog having lesser sialic acid content. Native FLINT possesses one N-linked site and one O-linked site, whereas the analogs possess at least one additional N-linked site and/or O-linked sites.

FLINT Analog Isoforms

The subject invention provides compositions of FLINT analog isoforms. The specific isoforms of FLINT obtained in accordance with the present invention, and their properties, may vary depending upon the source of the starting material. In a preferred embodiment, the invention relates to a FLINT isoform composition having an average sialic acid content of about 0.5 sialic acid residues per molecule of FLINT analog; alternatively, an average of about 1.0 sialic acids per molecule of FLINT analog; alternatively, an average of about 1.5 sialic acids per molecule of FLINT analog; alternatively, an average of about 2.0 sialic acids per molecule of FLINT analog; alternatively, an average of about 2.5 sialic acids per molecule of FLINT analog; alternatively, an average of about 3.0 sialic acids per molecule of FLINT analog; alternatively, an average of about 3.5 sialic acids per molecule of FLINT analog; alternatively, an average of about 4.0 sialic acids per

molecule of FLINT analog; alternatively, an average of about 4.5 sialic acids per molecule of FLINT analog; alternatively, an average of about 5.0 sialic acids per molecule of FLINT analog; alternatively, an average of about 5.5 sialic acids per molecule of FLINT analog; alternatively, an average of about 6.0 sialic acids per molecule of FLINT analog; alternatively, an average of about 6.5 sialic acids per molecule of FLINT analog; alternatively, an average of about 7.0 sialic acids per molecule of FLINT analog; alternatively, an average of about 7.5 sialic acids per molecule of FLINT analog; alternatively, an average of about 8.0 sialic acids per molecule of FLINT analog; alternatively, an average of about 8.5 sialic acids per molecule of FLINT analog; alternatively, an average of about 9.0 sialic acids per molecule of FLINT analog; alternatively, an average greater than about 9.0 sialic acids per molecule of FLINT analog.

The compositions of the invention, defined in terms of average sialic acid content, may comprise different FLINT analog isoforms, in other words, a composition of heterogeneous isoforms. For example, an N-linked site theoretically can accommodate 0, 1, 2, 3, or 4 sialic acids per molecule of analog while an O-linked site can theoretically accommodate 0, 1, or 2 sialic acids per molecule of analog. Thus, a composition having an average sialic acid content of about 1 could comprise multiple sialic acid isoforms. For example, each molecule of analog could independently have 1 sialic acid at each N-linked site or 1 sialic acid at the O-linked site, to give an average sialic acid content of 1. Alternatively, molecules with more than 1 sialic acid per molecule of analog could comprise the composition, so long as the average sialic acid content of 1

was maintained. Compositions of the invention comprise any combination of such isoforms that fall with a particular scope of the average sialic acid content. Thus, in one embodiment the compositions of the invention comprise a mixture of isoforms having an average number of sialic acids per FLINT analog molecule.

The invention also provides methods for preparing FLINT analog isoform compositions. These methods include isolation by techniques such as preparative isoelectric focusing, ion exchange chromatography, chromatofocusing.

In general, ion exchange chromatography and chromatofocusing involve application of either conditioned medium containing FLINT analog, or purified material, to a column resin under conditions that permit binding of some or all of the FLINT analog isoforms to the resin. It is preferable to apply the protein to the column at about pH 5. After washing the column with buffer at about pH 5, FLINT analog isoforms that remain bound on the ion exchange column are eluted by increasing the salt concentration of the buffer. For chromatofocusing, isoforms are eluted from the column by a gradient of decreasing pH, or by washing the column with a high concentration of salt.

FLINT analog molecules have N-linked or O-linked oligosaccharide structures which may limit the sialic acid content of the molecule. For example, tetra-antennary (four-branched) N-linked oligosaccharides provide four possible sites for sialic acid attachment, while bi- and triantennary oligosaccharide chains, which can substitute for the tetra-antennary form at asparagine-linked sites, commonly have only two or three sialic acids attached. O-linked oligosaccharides commonly provide two sites for sialic acid attachment.

Thus, native FLINT molecules can accommodate a total of 8 sialic acid residues provided the single N-linked oligosaccharides is tetra-antennary. Analogs would provide 0, 1, 2, 3, or 4 additional sialic acids per additional N-linked site, and 0, 1, or 2 additional sialic acids per additional O-linked site. The N-linked oligosaccharides of FLINT contain sialic acid in both an α-2,3 and an α-2,6 linkage to galactose (Takeuchi et al. J. Biol. Chem. 263, 3657(1988)). Typically the sialic acid in the α-2,3 linkage is added to galactose on the mannose α-1,6 branch, and the sialic acid in the α-2,6 linkage is added to the galactose on the mannose α-1,3 branch. The enzymes that add these sialic acids (β-galactoside α-2,3 sialyltransferase and β-galactoside α-2,6 sialyltransferase) are most efficient at adding sialic acid to the mannose α-1,6 and mannose α-1,3 branches respectively.

Mammalian cell cultures may be screened for cells that preferentially add tetra-antennary chains to recombinant FLINT analog, thereby maximizing the number of sites for sialic acid attachment. Dihydrofolate reductase (DHFR) deficient Chinese Hamster Ovary (CHO) cells are commonly used for the production of recombinant glycoproteins including recombinant FLINT. These cells do not express the enzyme β-galactoside α-2,6 sialyltransferase, and therefore do not add sialic acid in the α-2,6 linkage to N-linked oligosaccharides of glycoproteins produced in these cells. (Mutsaers et al. Eur. J. Biochem. 156, 651 (1986); Takeuchi et al. J. Chromatogr. 400, 207 (1987)). Consequently, recombinant FLINT produced in CHO cells lacks sialic acid in the 2,6 linkage to galactose (Sasaki et al. (1987), supra; Takeuchi et al. (1987), supra). Therefore, in one embodiment

of the invention, FLINT analog isoforms are made in CHO cells that are transfected with a functional β-galactoside α-2,6 sialyltransferase gene to give incorporation of sialic acid in α-2,6 linkage to galactose. See Lee et al. J. Biol. Chem. 264, 13848 (1989), hereby incorporated by reference, for a disclosure of techniques for creating modified CHO cells, or other mammalian host cells.

Enzymatically Enhanced Sialylation of FLINT

Also contemplated by the present invention is a method for enhancing the sialylation of FLINT analog isoforms by enzymatic modification *in vitro*.

The circulatory lifetime of glycoproteins such as FLINT analog in blood is highly dependent on the composition and structure of N-linked oligosaccharides. In general, maximal plasma half-life of a glycoprotein requires that its N-linked carbohydrate groups terminate in the sequence NeuAcGalGlcNAc. Without a terminal sialic acid residue (NeuAc), a glycoprotein is rapidly cleared from the blood by receptors that recognize the exposed Gal residues. For this reason, ensuring high sialylation of therapeutic proteins such as FLINT analog is important for commercial development.

Although much is known about the complexity of carbohydrate structures on glycoproteins, attempts to specify post-translational glycosylation in cultured cells have not kept pace with advances in technology for gene expression, and therefore, incomplete glycosylation of secreted recombinant glycoproteins, including FLINT analogs, is common. One solution to this problem is to use isolated

glycosyltransferases to complete carbohydrate chains *in vitro*.

Optimal glycosylation may be difficult to achieve using mammalian cell culture systems. Under conditions of large scale growth, overproduction of a protein backbone comprising a glycoprotien can exceed the host cells capacity to achieve full sialylation.

A method of the present invention comprises use of sialyltransferase to add sialic acids to an acceptor site(s) on FLINT analog, preferably said site(s) having a galactosyl unit. The method for enhancing sialylation of FLINT follows that disclosed in U.S. Patent No. 6,030,815, herein incorporated by reference. Essentially, the method comprises the steps of adding sialyltransferase to a sample of FLINT analog and a catalytic amount of a CMP-sialic acid synthetase, a sialic acid, CTP, and a soluble divalent metal cation, including Mn^{+2}, Mg^{+2}, Ca^{+2}, Co^{+2}, and Zn^{+2}. Preferably, the divalent ion concentration is maintained between 2mM and 75 mM. Alternatively, the reaction may further comprise a CMP-sialic acid recycling system, as disclosed in U.S. Patent 6,030,815. A commercially available system for carrying out the reactions, GlycoAdvance®, is available from Neose Technologies, Inc. (Horsham, PA).

In one embodiment of the invention, FLINT analog is produced recombinantly in a suitable mammalian cell type, for example, CHO cells, by transfection with a suitable vector for expressing FLINT analog. Culture supernatants containing FLINT analog are concentrated and processed using the GlycoAdvance® system, or comparable commercial or non-commercial system. FLINT analog having enhanced sialylation is recovered using standard purification techniques.

Also comprehended by the invention are pharmaceutical compositions comprising a therapeutically effective amount of a FLINT analog isoform, having an average sialic acid content of about 0.5 sialic acid residues per molecule of FLINT analog; alternatively, an average of about 1.0 sialic acids per molecule of FLINT analog; alternatively, an average of about 1.5 sialic acids per molecule of FLINT analog; alternatively, an average of about 2.0 sialic acids per molecule of FLINT analog; alternatively, an average of about 2.5 sialic acids per molecule of FLINT analog; alternatively, an average of about 3.0 sialic acids per molecule of FLINT analog; alternatively, an average of about 3.5 sialic acids per molecule of FLINT analog; alternatively, an average of about 4.0 sialic acids per molecule of FLINT analog; alternatively, an average of about 4.5 sialic acids per molecule of FLINT analog; alternatively, an average of about 5.0 sialic acids per molecule of FLINT analog; alternatively, an average of about 5.5 sialic acids per molecule of FLINT analog; alternatively, an average of about 6.0 sialic acids per molecule of FLINT analog; alternatively, an average of about 6.5 sialic acids per molecule of FLINT analog; alternatively, an average of about 7.0 sialic acids per molecule of FLINT analog; alternatively, an average of about 7.5 sialic acids per molecule of FLINT analog; alternatively, an average of about 8.0 sialic acids per molecule of FLINT analog; alternatively, an average of about 8.5 sialic acids per molecule of FLINT analog; alternatively, an average of about 9.0 sialic acids per molecule of FLINT analog; alternatively, an average greater than about 9.0 sialic acids per molecule of FLINT analog, together with a suitable diluent, adjuvant and/or carrier

useful in therapeutic applications. A therapeutically effective amount as used herein refers to that amount which provides therapeutic effect for a given condition and administration regimen. The administration of FLINT analog isoforms is preferably by the intravenous route.

Therapeutic Applications

The clinical utility for the FLINT analog isoforms of the invention is expected to be substantial. Active FLINT analog isoforms inhibit the binding of Fas to FasL and LIGHT to LTBR and TR2/HVEM receptors, and can be used to treat or prevent a disease and/or condition that may be associated with such binding.

Many diseases and/or conditions involving FasL/Fas are potentially amenable to therapy with FLINT analog isoforms. Examples of suitable diseases and/or conditions include the following.

Inflammatory/autoimmune diseases - Rheumatoid arthritis, inflammatory bowel disease, graft-versus-host disease, insulin-dependent diabetes, SIRS/sepsis/MODS, pancreatitis, psoriasis, multiple sclerosis, Hashimoto's thyroiditis, Grave's disease, transplant rejection, SLE, autoimmune gastritis, fibrosing lung disease.

Infectious diseases - HIV-induced lymphopenia, fulminant viral hepatitis B/C, chronic hepatitis/cirrhosis, H. pylori-associated ulceration.

Ischemia/Re-perfusion conditions - Acute coronary syndrome, acute myocardial infarction, congestive heart failure, atherosclerosis, acute cerebral ischemia/infarction, brain/spinal cord trauma, organ preservation during transplant

Other treatments include cytoprotection during cancer treatment, adjuvant to chemotherapy, Alzheimer's, chronic glomerulonephritis, osteoporosis, TTP/HUS, aplastic anemia, myelodysplasia. Also of interest are treatment and prevention of acute lung injury (ALI)/acute respiratory distress syndrome (ARDS); Ulcerative colitis; and Crohn's disease.

Other diseases for which FLINT analog isoforms are therapeutically useful include rheumatoid arthritis (Elliott *et al., Lancet* 344:1105-10 (1994)), fibroproliferative lung disease, fibrotic lung disease, HIV (Dockrell *et al., J. Clin. Invest.* 101:2394-2405 (1998)), Ischemia (Sakurai et al. 1998 Brain Res 797:23-28), Brain trauma/injury (Ertel et al. 1997 J Neuroimmunol 80:93-6), chronic renal failure (Schelling et al. 1998 Lab Invest 78:813-824), Graft-vs-Host Disease (GVHD) (Hattori et al. 1998 Blood 11:4051-4055), Cutaneous inflammation (Orteu at al. 1998 J Immunol 161:1619-1629), Vascular leak syndrome (Rafi et al. 1998 J Immunol 161:3077-3086), Helicobacter pylori infection (Rudi et al. 1998 J Clin Invest 102:1506-1514), Goiter (Tamura et al. 1998 Endocrinology 139:3646-3653), Atherosclerosis (Sata and Walsh, 1998 J Clin Invest 102:1682-1689), IDDM (Itoh et al. 1997 J Exp Med 186:613-618), Osteoporosis (Jilka et al. 1998 J Bone Min Res 13:793-802), Crohn's Disease (van Dullemen et al. 1995 Gastroenterology 109:129-35), organ preservation and transplant (graft) rejection (Lau et al. 1996 Science 273:109-112), Sepsis (Faist and Kim. 1998 New Horizons 6:S97-102), Pancreatitis (Neoptolemos et al. 1998 Gut 42:886-91), Cancer (melanoma, colon and esophageal) (Bennett et al. 1998 J Immunol 160:5669-5675), Autoimmune disease (IBD, psoriasis, Down's Syndrome (Seidi *et al., Neuroscience*

Lett. 260:9 (1999), multiple sclerosis (D'Souza et al. 1996 J Exp Med 184:2361-70), Alzheimer's Disease; End-stage renal disease (ESRD); mononulceosis; EBV; Herpes; antibody dependent sytotoxicity; hemolytic and hypercoagulation disorders such as vascular bleeds, DIC (disseminated intervascular coagulation), eclampsia, HELLP (preeclampsia complicated by thrombocytopenia, hemolysis and disturbed liver function), HITS (heparin induced thromobcytopenia), HUS (hemolytic uremic syndrome), and preeclampsia; hematopoeitic disorders such as aplastic anemia, thrombocytopenia (TTP) and myelodysplasia; and hemolytic fever caused, for example, by E.bola.

In the case of organ preservation in preparation for harvesting, for instance, a FLINT analog isoform is useful prophylactically to prevent the apoptosis associated with ischemia reperfusion injury to the organ once it is removed from the donor. Suitable media for this purpose are known, for example, the media disclosed in EP 0356367 A2. The method may also include treating the transplant recipient with FLINT analog isoform prior to and/or after the transplant surgery.

There is evidence that ARDS may be mediated by soluble FasL/Fas interaction in humans (Matute-Bello et al., J. Immunol. 163, 2217-2225, 1999). FLINT, by binding to FasL, could inhibit FasL-mediated apoptosis of pneumocytes and/or endothelial cells, thus inhibiting or preventing the progression from acute inflammatory insult to ALI, and from ALI to ARDS.

Therefore, in another embodiment, the present invention relates to the use of a FLINT analog isoforms to inhibit and/or treat ALI and/or ARDS comprising the administration

of a therapeutically effective amount of analog isoform to a person in need thereof.

In another embodiment, the present invention relates to the use of a FLINT analog isoform to treat and/or inhibit chronic obstructive pulmonary disease (COPD) in a patient in need thereof by administering a therapeutically effective amount of FLINT analog isoform.

In another embodiment the present invention relates to the use of a FLINT analog isoform to inhibit and/or treat pulmonary fibrosis (PF). For example, FLINT analog isoform can be administered acutely at the time of an inflammatory insult to the lung (e.g. during bleomycin treatment) to prevent PF from occurring.

A "subject" is a mammal in need of treatment, preferably a human, but can also be an animal in need of veterinary treatment, e.g., domestic animals (e.g., dogs, cats, and the like), farm animals (e.g., cows, sheep, pigs, horses, and the like) and laboratory animals (e.g., rats, mice, guinea pigs, and the like).

An "effective amount" of FLINT analog isoform is an amount which results in a sufficient inhibition of one or more processes mediated by the binding of Fas to FasL or LIGHT to LTBR and/or TR2/HVEM so as to achieve a desired therapeutic or prophylactic effect in a subject with a disease or condition that may be associated with aberrant Fas/FasL binding and/or LIGHT mediated binding. Alternatively, an "effective amount" of FLINT analog isoform is a quantity sufficient to achieve a desired therapeutic and/or prophylactic effect in a subject with inflammation caused by FasL-induced neutrophil activation or any of the other aforementioned diseases associated with aberrant FasL activity.

A "desired therapeutic and/or prophylactic effect" in a subject with a disease or condition includes the amelioration of symptoms, or delay in onset of symptoms, associated with such disease. Alternatively, a "desired therapeutic and/or prophylactic effect" includes an increased survival rate or increased longevity for the subject with the disease.

The amount of FLINT analog isoform administered to the individual will depend on the type and severity of the disease and on the characteristics of the individual, such as general health, age, sex, body weight and tolerance to drugs. It will also depend on the degree, severity and type of disease. The skilled artisan will be able to determine appropriate dosages depending on these and other factors.

As a general proposition, the total pharmaceutically effective amount of the FLINT analog isoform molecules of the present invention administered parenterally per dose will be in the range of about 1 μg/kg/day to 10 mg/kg/day of patient body weight, particularly 2 mg/kg/day to 8 mg/kg/day, more particularly 2 mg/kg/day to 4 mg/kg/day, even more particularly 2.2 mg/kg/day to 3.3 mg/kg/day, and finally 2.5 mg/kg/day, although, as noted above, this will be subject to therapeutic discretion. More preferably, this dose is at least 0.01 mg/kg/day. If given continuously, a FLINT analog isoform of the present invention is typically administered at a dose rate of about 1 μg/kg/hour to about 50 μg/kg/hour, either by 1-4 injections per day or by continuous subcutaneous infusions, for example, using a mini-pump. An intravenous bag solution may also be employed. The length of treatment needed to observe changes and the interval following treatment for responses to occur appears to vary depending on the desired effect.

Pharmaceutical compositions comprising FLINT analog isoform(s) of the present invention may be administered orally, rectally, intracranially, parenterally, intracisternally, intravaginally, intraperitoneally, topically (as by powders, ointments, drops or transdermal patch), transdermally, intrathecally, bucally, or as an oral or nasal spray. By "pharmaceutically acceptable carrier" is meant a non-toxic solid, semisolid or liquid filler, diluent, encapsulating material or formulation auxiliary of any type. The term "parenteral" as used herein includes, but is not limited to, modes of administration which include intravenous, intramuscular, intraperitoneal, intrasternal, subcutaneous and intraarticular injection, infusion and implants comprising FLINT analog isoforms.

The FLINT analog isoforms of the present invention are also suitably administered by sustained-release systems. Suitable examples of sustained-release compositions include semi-permeable polymer matrices in the form of shaped articles, e.g., films, or microcapsules. Sustained-release matrices include polylactides (U.S. Pat. No. 3,773.919, EP 58,481), copolymers of L-glutamic acid and gamma-ethyl-L-glutamate (Sidman, U. et al., *Biopolymers 22*:547-556 (1983)), poly (2-hydroxyethyl methacrylate) (R.Langer et al., *J. Biomed. Mater. Res. 15*:167-277 (1981), and R. Langer, *Chem. Tech. 12*:98-105 (1982)), ethylene vinyl acetate (R. Langer et al., Id.) or poly-D-(-)-3-hydroxybutyric acid (EP 133,988). Other sustained-release compositions also include liposomally entrapped FLINT analog isoform. Such liposomes are prepared by methods known per se: DE 3,218,121; Epstein et al., Proc. Natl. Acad. Sci. (USA) 82:3688-3692 (1985); Hwang et al., Proc. Natl. Acad. Sci. (USA) 77:4030-4034 (1980); EP 52,322; EP 36,676; EP

88,046; EDP 143,949; EP 142,641; Japanese Pat. Appl. 83-118008; U.S. Pat. Nos. 4,485,045 and 4,544,545; and EP 102,324. Ordinarily, the liposomes are of the small (about 200-800 Angstroms) unilamellar type in which the lipid content is greater than about 30 mol. percent cholesterol, the selected proportion being adjusted for the optimal TNFR polypeptide therapy.

For parenteral administration, the FLINT analog isoforms of the present invention are formulated generally by mixing at the desired degree of purity, in a unit dosage injectable form (solution, suspension, or emulsion), with a pharmaceutically acceptable carrier, i.e., one that is non-toxic to recipients at the dosages and concentrations employed and is compatible with other ingredients of the formulation. For example, the formulation preferably does not include oxidizing agents and other compounds that are known to be deleterious to polypeptides.

The FLINT analog isoforms of the present invention are typically formulated in suitable vehicles at a concentration of about 0.1 mg/ml to 100 mg/ml, preferably 1-10 mg/ml, at a pH of about 3 to 8. It will be understood that the use of certain excipients, carriers, or stabilizers will result in the formation of salts of the FLINT analog molecules of the present invention.

The invention also provides a pharmaceutical pack or kit comprising one or more containers filled with one or more of the ingredients of the pharmaceutical compositions of the invention. Associated with such container(s) can be a notice in the form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals or biological products, which notice reflects approval by the agency of manufacture, use or sale for human

administration. In addition, the FLINT analog isoforms of the present invention may be employed in conjunction with other therapeutic compounds.

The following examples are offered to more fully illustrate the invention, but are not to be construed as limiting the scope thereof.

EXAMPLE 1

Isolation of Recombinant FLINT Analog A12N Isoforms

A bicistronic expression vector was constructed by inserting into mammalian expression vector pGTD (Gerlitz, B. et al., 1993, Biochemical Journal 295:131) a PCR fragment encoding an "internal ribosome entry site"/enhanced green fluorescent polypeptide (IRES/eGFP). The new vector, designated pIG3, contains the following elements: the E1a-responsive GBMT promoter (D. T. Berg et al., 1993 BioTechniques 14:972; D.T. Berg et al., 1992 Nucleic Acids Research 20:5485); a multiple cloning site (MCS); the IRES sequence from encephalomyocarditis virus (EMCV); the eGFP coding sequence (Cormack, et al., 1996 Gene 173:33, Clontech); the SV40 small "t" antigen splice site/poly-adenylation sequences; the SV40 early promoter and origin of replication; the murine dihydrofolate reductase (dhfr) coding sequence; and the ampicillin resistance gene and origin of replication from pBR322.

Based on the human FLINT cDNA sequence (e.g. SEQ ID NO:3), forward and reverse PCR primers were synthesized bearing *BclI* restriction sites at their respective 5'ends. These primers were used to amplify the FLINT cDNA. The FLINT cDNA orientation and nucleotide sequence was confirmed by restriction digest and double stranded sequencing of the insert. The approximately 900 base pair amplified FLINT

analog PCR product was digested with restriction endonucleases NheI and XbaI, respectively, to generate a fragment bearing NheI and XbaI sticky ends. This fragment was subsequently ligated into a unique XbaI site of pIG3 to generate recombinant plasmid pIG3-FLINT.

The recombinant pIG3-FLINT plasmid carries the FLINT gene and encodes resistance to methotrexate. In vitro mutagenesis processes were followed to change Ala 12 to Asn, in order to introduce an additional N-linked glycosylation site as described elsewhere in this disclosure. AV12 RGT18 cells are transfected using a calcium phosphate procedure with the recombinant pIG vector. Cells resistant to 250 nM methotrexate are selected and pooled. The pool of resistant clones is subjected to fluorescence-assisted cell sorting (FACS), and cells having fluorescence values in the top 5% are sorted into a pool, and as single cells. High fluorescence pools are subjected to two successive sorting cycles. Pools and individual clones from the first and second cycles are analyzed for FLINT analog production by ELISA. Pools or clones expressing FLINT analog at the highest level are used for scale-up and FLINT analog purification.

Large-scale production of FLINT analog A12N is carried out by growing stable clones of AV12 RGT 18 cells transfected with an expression vector that expresses A12N. After reaching confluence, cells are further incubated for 2-3 more days to secrete maximum amount of FLINT analog into the growth medium. Medium containing FLINT analog is adjusted to 0.1 % CHAPS and concentrated in an Amicon ProFlux M12 tangential filtration system to 350 ml. The concentrated medium is adjusted to pH 6.0 and passed over a SP Sepharose Fast Flow (Pharmacia, 500 ml) at a flow rate of

7 ml/min. The column is washed with buffer A (20 mM MOPS, 0.1 % CHAPS, pH 6.0) until the absorbance (280 nm) returns to baseline and bound polypeptides are eluted with a linear gradient from 0 to 1 M NaCl (in buffer A) developed over four column volumes. Fractions containing FLINT only are pooled and passed over Vydac C4 column (100 ml) equilibrated with 0.1 % TFA/H_2O at a flow rate of 10 ml/min. This material is passed over a 16/60 Superdex 200 sizing column (Pharmacia) equilibrated with PBS, 0.5 M NaCl, pH 7.4. Fractions containing FLINT analog are analyzed by SDS-PAGE, and the N-terminal sequence of the purified polypeptide confirmed to be FLINT.

In an alternative purification scheme, concentrated and clarified medium containing FLINT analog is passed over Blue Sepharose DAC and eluted with 7M urea, 1M NaCl, pH 8. Eluted material is further purified on CG71 reverse phase chromatography and eluted with 35% acetonitrile, 0.6 M NaCl, pH 7.4. The eluted material is purified further on SP650M cation exchange with elution in 30% acetonitrile, pH 2.5. Following this step, eluted material is subjected to solvent exchange by TFF into buffer for bulk freezer storage.

FLINT A12N has two potential N-linked glycosylation sites, at Asn 12 and Asn144 of SEQ ID NO:1. To characterize the oligosaccharide structure of A12N produced in AV12 cells, samples of intact FLINT analog A12N, and FLINT A12N that had been treated with neuraminidase and HEXase II to release terminal GalNAc and GlcNAc residues (desialylated FLINT), is analyzed by capillary HPLC/ESI-MS. The oligosaccharides released by this treatment are labeled with 2-aminobenzamide and analyzed by weak anion exchange (WAE) HPLC and by LC mass spectrophotometry (LC/MS).

EXAMPLE 2

Oligosaccharide Profile of FLINT A12N by HPLC/ESI-MS and Fluorescence HPLC

Recombinant A12N FLINT is expressed in AV12 cells and purified as in Example 1. Intact FLINT A12N is directly analyzed by a capillary HPLC/ESI-MS, or treated by neuraminidase, or HEXase II, followed by HPLC/ESI-MS. The oligosaccharide structures are calculated based on the obtained masses and the expected FLINT analog peptide backbone mass. Fluorescence labeled FLINT analog oligosaccharides are fractionated by weak anion exchange (WAE) HPLC. The fractions are collected and identified by liquid chromatography/mass spectrophotometry (LC/MS).

Neuraminidase, HEXase II Treatment of A12N Ten microliters of a solution containing FLINT A12N (~0.43 mg/ml in PBS, 0.5M NaCl) is mixed with 8 uL of 50 mM NaOAc buffer, pH 5.2, and 2 uL of neuraminidase solution (1 unit/mL). The mixture is incubated at 37°C for 2 hours. Seven microliters of the mixture is used for capillary HPLC/MS analysis and two microliters of HEXase II enzyme solution (Glyko, Inc.) is added to the remaining solution, which is incubated at 37°C for 3 hours before HPLC/MS analysis.

Weak Anion Exchange (WAE) HPLC of Fluorescence labeled Oligosaccharides. A 200 ul Aliquot of thawed FLINT A12N solution containing approximately 0.2 mg of protein is mixed with 60 mg urea, 17.6 ul of 3 M Tris buffer (pH 8.0) and 3 ul of 50 mg/mL dithiothreitol and the mixture was incubated at 37°C for 10 min. The sample is alkylated by adding 5 ul of 100 mg/mL iodoacetic acid solution and incubating at ambient temperature in the dark for 10 min. Samples are desalted on a disposable gel filtration column and oligosaccharides released by treatment with 1 unit N-

glycosidase F solution at 37°C for 2 hours. The deglycosylated protein is precipitated by adjusting pH with 10% (v/v) acetic acid solution. A 300 ul aliquot of oligosaccharide solution is dried and labeled with 2-aminobenzamide dye. After the excess dye is removed using a P-2 spin column, the labeled oligosaccharide solution is analyzed by WAE-HPLC with a fluorescence detector. Desialylation of WAE HPLC fractions of 2-AB labeled FLINT Oligosaccharides. Each of the collected WAE HPLC fractions is transferred into two vials and dried using a centrifugation vacuum system. Fifteen microliters (containing 2.5 munits neuraminidase) of 15 mM NaOAc buffer, pH 5.2 is added to one vial for each fraction. Five microliters of the mixture is used for LC/MS analysis after incubation at room temperature for 10 to 15 hours. For intact oligosaccharide fraction, fifteen microliters of H_2O is added into each vial and 5 ul of the solution is used for LC/MS analysis.

Capillary HPLC/ESI-MS. A Beckman System Gold equipped with a Model 126 solvent delivery module is used. The HPLC buffer (A: 0.15% formic acid in H_2O and B: 0.12% formic acid in ACN) is pumped through a T split, in which a Zorbax 300SB C18, 2.1x150 mm column is attached on one exit and a manual injection valve, Vydac capillary column (C18, 0.3x150 mm) and a API UV detector (785A) at the other exit. The HPLC stream from the capillary directly passes to the mass spectrometer through a fused silica transfer line. Beckman solvent deliver system is pumped at 0.2 ml/min with the following gradient

Time (min)	0	2	42	43	45	46	57

Buffer B%	10	10	45	90	90	10	10

After the split, about 5-6 ul/min of HPLC stream is diverted to the capillary column. About 2 ug FLINT A12N is injected to capillary column per run. API UV detector is set lambda = 214 nm and the data stored in HP1000. A PE Sciex API III mass spectrometer equipped with an articulated ionspray source is used in these studies under the condition of CC 1, OR 55 V and ISV 4800 V, Q1 scan from 900 to 1500 or 1000 to 1400, 0.33 or 0.20/step, 2 to 3 ms dwell time and 6 sec/scan. For oligosaccharide fraction analysis, a short solvent gradient program is used.

FLINT A12N has two potential N-glycosylation sites. The theoretical molecular weight of the protein alone is 29736.9 Da. The mass spectrum and reconstructed mass spectrum of FLINT A12N are analyzed for the molecular weights of the primary signals.

Based on these experimental results, the primary A12N glycoforms are assigned.

The fluorescence-labeled FLINT A12N oligosaccharides are fractionated into multiple peaks on WAE HPLC according to their negative charge number. The primary structures of oligosaccharides are identified in each fraction.

Because FLINT A12N contains two glycosylation sites, sialic acid content can be calculated based on the results of WEA HPLC as follows:

Sialic Acid Content = $\sum$ sialylation degree of fraction $\times$ percentage of fraction/100

Sialic acid content may also be determined by a modification of the procedure of Jourdian et al. J. Biol. Chem. 246, 430 (1971). The sialic acid residues are cleaved from glycoproteins by hydrolysis with 0.35M sulfuric acid at

80° C for 30 minutes and the solutions neutralized with sodium hydroxide prior to analysis. In order to estimate the amount of protein present, a Bradford protein assay (Bradford Anal. Biochem. 72, 248 (1976)) using recombinant FLINT as standard is performed using the assay reagents and the micro-method procedure supplied by Bio-Rad.

EXAMPLE 3

Effect of Higher Sialylation on Clearance of A12N FLINT

The pharmacokinetics of different lots of FLINT A12N are examined for the influence of sialic acid content on the clearance in male Cynomolgus monkeys. Sialic acid content may be determined as in Example 2, based on LC/MS and oligosaccharide profiling.

Lots of A12N are administered as a single intravenous bolus dose (0.5 mg/kg) and blood samples obtained over a 48 h period after dosing.

Plasma samples from treated animals are analyzed for concentrations of FLINT(A12N) using a sandwich ELISA method employing affinity purified rabbit polyclonal anti-FLINT antibodies.

After intravenous administration, lots of A12N are analyzed for clearance from the plasma of treated animals. The data indicate that the extent of terminal sialylation on the carbohydrate moieties present on FLINT A12N have an influence on the clearance kinetics of the compound after administration by the intravenous route. The increased rate of clearance of poorly sialylated molecules from the circulation is most likely mediated through hepatic asialoglycoprotein receptors via exposed terminal galactose residues.

EXAMPLE 4

Effect of Higher Sialylation on Clearance of FLINT Analogs

The pharmacokinetics of multiple lots FLINT A12N are examined for the influence of sialic acid content on clearance in mammals, for example, male Cynomolgus monkeys. Oligosaccharide and sialic acid profiling using LC-MS is used to ascertain sialylation differences between lots.

Lots of FLINT A12N having differing average sialic acid content are administered as a single intravenous bolus dose to Cynomolgus monkeys (0.5 mg/kg), and blood samples are obtained over a 48 hour period after dosing.

Plasma samples from treated animals are analyzed for concentrations of FLINT A12N using a sandwich ELISA method employing affinity purified rabbit polyclonal anti-FLINT antibodies. The capture antibody recognizes the N-terminal portion of FLINT. The sandwich antibody is a biotinylated polyclonal antibody which recognizes the C-terminal portion of FLINT.

The effect of sialylation is determined by measuring the clearance kinetics of the A12N analog after administration by the intravenous route.

EXAMPLE 5

Fractionation of Recombinant FLINT Analog Isoforms Using a Low pH Gradient on SP-Sepharose

FLINT analog isoforms are separated using a gradient of decreasing pH and increasing ionic strength. For example, concentrated and filtered FLINT A12N-containing medium prepared as in Example 1 is loaded onto a column of SP-Sepharose at a ratio of approximately 20 mg total protein/mL gel. The column is then washed with approximately three column volumes of 20 mM MOPS, pH 5.5. FLINT A12N isoforms

are eluted from the column using a gradient starting with 20 mM MOPS, pH 5.5 and running to 20 mM MOPS, 600 mM NaCl, pH 5.5. The total volume of the gradient is approximately 40 column volumes.

EXAMPLE 6

Analogs of FLINT Having Additional Glycosylation Sites

FLINT analogs having additional carbohydrate attachment sites are described elsewhere in this specification. Mutations are introduced into the native FLINT cDNA using well known *in vitro* mutagenesis techniques.

FLINT variant A12N was constructed by mutagenic PCR starting from a wild-type FLINT template. *See e.g.* Saiki R. K. *et al. Science 239:*487-491 (1988), and "Current Protocols in Molecular Biology", Vol 1, section 8.5.7 (John Wiley and Sons, Inc. publishers), the entire contents of which are herein incorporated by reference.

The mutagenic PCR process involves a "SOEing" reaction (i.e. Strand Overlap Extension) to create specific mutations in the native FLINT template for the purpose of changing the amino acid sequence at position 12, and further for introducing restriction enzyme tags for identification purposes.

Generally, SOEing reactions require the use of four primers, two in the forward orientation (termed A, SEQ ID NO:5, and C, SEQ ID NO:7) and two in the reverse orientation (termed B, SEQ ID NO:6 and D, SEQ ID NO:8). The SOEing reaction amplifies a nucleic acid sequence (e.g. gene sequence) in two stages. The first step amplifies a portion of the gene by performing an A to B reaction followed by a separate C to D reaction. In constructing the A12N mutant, the B and C primers were targeted to the same area of the

gene but on opposite strands. Mismatch priming from both oligonucleotide primers produces the mutation. After these two reactions were completed, the products were isolated and mixed for use as template for the A to D reaction, which yields the desired mutated product.

The primers involved in the cloning of A12N were:
CF119 (A): gag cta gcc acc atg agg gcg ctg gag ggg cca ggc ctg tcg ctg

CF120(B): GTC TCG TTG TCC CGC CAT GGG TAG GTG GGT GTT TCT GCC ACT CCG CGT ACA G

CF121(C): ggc aga aac acc cac cta ccc atg gcg gga caa cga gac agg gga gcg gct g

CF122(D): GTC GAT GAC GGC ACG CTC ACA CTC CTC AGC TCC TGG TAC CCT GGT GCT G

The amplified fragment carrying the A12N mutation was sub-cloned using an 5′ NheI site (GAGCTA) and a 3′ KpnI site (GAGGAG). The native FLINT sequence has a naturally occurring internal KpnI site around amino acid position 176. The amplified mutant fragment was incorporated into the full length FLINT sequence as follows:
First, the amplified fragment was placed into an intermediate vector, pCR2.1- TOPO, which utilizes the adenine overhangs established after PCR for ligation. And second, the mutated fragment was used to replace the corresponding segment in the wild type FLINT gene by directional ligation.

While the invention has been described in what is considered to be its preferred embodiments, it is not to be limited to the disclosed embodiments, but on the contrary,

is intended to cover various modifications and equivalents included within the spirit and scope of the appended claims, which scope is to be accorded the broadest interpretation so as to encompass all such modifications and equivalents.

We Claim:

1. A composition comprising a FLINT analog isoform, said analog consisting essentially of SEQ ID NO:3 wherein a change in sequence results in an additional glycosylation site, said composition having an average sialic acid content of about 0.5 sialic acids per molecule of FLINT analog.
2. A composition comprising a FLINT analog isoform, said analog consisting essentially of SEQ ID NO:3 wherein a change in sequence results in an additional glycosylation site, said composition having an average sialic acid content of about 1.0 sialic acids per molecule of FLINT analog.
3. A composition comprising a FLINT analog isoform, said analog consisting essentially of SEQ ID NO:3 wherein a change in sequence results in an additional glycosylation site, said composition having an average sialic acid content of about 1.5 sialic acids per molecule of FLINT analog.
4. A composition comprising a FLINT analog isoform, said analog consisting essentially of SEQ ID NO:3 wherein a change in sequence results in an additional glycosylation site, said composition having an average sialic acid content of about 2.0 sialic acids per molecule of FLINT analog.
5. A composition comprising a FLINT analog isoform, said analog consisting essentially of SEQ ID NO:3 wherein a change in sequence results in an additional glycosylation site, said composition having an average sialic acid content of about 2.5 sialic acids per molecule of FLINT analog.

6. A composition comprising a FLINT analog isoform, said analog consisting essentially of SEQ ID NO:3 wherein a change in sequence results in an additional glycosylation site, said composition having an average sialic acid content of about 3.0 sialic acids per molecule of FLINT analog.
7. A composition comprising a FLINT analog isoform, said analog consisting essentially of SEQ ID NO:3 wherein a change in sequence results in an additional glycosylation site, said composition having an average sialic acid content of about 3.5 sialic acids per molecule of FLINT analog.
8. A composition comprising a FLINT analog isoform, said analog consisting essentially of SEQ ID NO:3 wherein a change in sequence results in an additional glycosylation site, said composition having an average sialic acid content of about 4.0 sialic acids per molecule of FLINT analog.
9. A composition comprising a FLINT analog isoform, said analog consisting essentially of SEQ ID NO:3 wherein a change in sequence results in an additional glycosylation site, said composition having an average sialic acid content of about 4.5 sialic acids per molecule of FLINT analog.
10. A composition comprising a FLINT analog isoform, said analog consisting essentially of SEQ ID NO:3 wherein a change in sequence results in an additional glycosylation site, said composition having an average sialic acid content of about 5.0 sialic acids per molecule of FLINT analog.
11. A composition comprising a FLINT analog isoform, said analog consisting essentially of SEQ ID NO:3 wherein a

change in sequence results in an additional glycosylation site, said composition having an average sialic acid content of about 5.5 sialic acids per molecule of FLINT analog.

12. A composition comprising a FLINT analog isoform, said analog consisting essentially of SEQ ID NO:3 wherein a change in sequence results in an additional glycosylation site, said composition having an average sialic acid content of about 6.0 sialic acids per molecule of FLINT analog.

13. A composition comprising a FLINT analog isoform, said analog consisting essentially of SEQ ID NO:3 wherein a change in sequence results in an additional glycosylation site, said composition having an average sialic acid content of about 6.5 sialic acids per molecule of FLINT analog.

14. A composition comprising a FLINT analog isoform, said analog consisting essentially of SEQ ID NO:3 wherein a change in sequence results in an additional glycosylation site, said composition having an average sialic acid content of about 7.0 sialic acids per molecule of FLINT analog.

15. A composition comprising a FLINT analog isoform, said analog consisting essentially of SEQ ID NO:3 wherein a change in sequence results in an additional glycosylation site, said composition having an average sialic acid content of about 7.5 sialic acids per molecule of FLINT analog.

16. A composition comprising a FLINT analog isoform, said analog consisting essentially of SEQ ID NO:3 wherein a change in sequence results in an additional glycosylation site, said composition having an average sialic acid

content of about 8.0 sialic acids per molecule of FLINT analog.

17. A composition comprising a FLINT analog isoform, said analog consisting essentially of SEQ ID NO:3 wherein a change in sequence results in an additional glycosylation site, said composition having an average sialic acid content of about 8.5 sialic acids per molecule of FLINT analog.

18. A composition comprising a FLINT analog isoform, said analog consisting essentially of SEQ ID NO:3 wherein a change in sequence results in an additional glycosylation site, said composition having an average sialic acid content of about 9.0 sialic acids per molecule of FLINT analog.

19. A composition comprising a FLINT analog isoform, said analog consisting essentially of SEQ ID NO:3 wherein a change in sequence results in an additional glycosylation site, said composition having an average sialic acid content of about 9.5 sialic acids per molecule of FLINT analog.

20. A pharmaceutical composition comprising a therapeutically effective amount of FLINT analog according to claim 2 and a pharmaceutically acceptable diluent, adjuvant or carrier.

SEQUENCE LISTING

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<120> FLINT Analog Glycoforms

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Cys Arg Arg Asp Ser Pro Thr Thr Cys Gly Pro Cys Pro Pro Arg His
         35                  40                  45

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Phe Ser Ala Ser Ser Ser Ser Ser Glu Gln Cys Gln Pro His Arg Asn
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Asp Thr Leu Cys Thr Ser Cys Thr Gly Phe Pro Leu Ser Thr Arg Val
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tac tgc aac gtc ctc tgc ggg gag cgt gag gag gag gca cgg gct tgc     339
Tyr Cys Asn Val Leu Cys Gly Glu Arg Glu Glu Glu Ala Arg Ala Cys
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cac gcc acc cac aac cgt gcc tgc cgc tgc cgc acc ggc ttc ttc gcg     387
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cac gct ggt ttc tgc ttg gag cac gca tcg tgt cca cct ggt gcc ggc     435
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Pro His Arg Asn Cys Thr Ala Leu Gly Leu Ala Leu Ile Val Pro Gly
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INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 02/00509

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C07K14/705 A61K38/17

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C07K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, MEDLINE, BIOSIS, CHEM ABS Data, SEQUENCE SEARCH

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 00 58465 A (BECKER GERALD WAYNE ;COHEN FREDRIC JAY (US); GONZALEZ DEWHITT PATR) 5 October 2000 (2000-10-05) cited in the application page 31, paragraph 1 -page 32, paragraph 1; examples 8,14,15	1-20
Y	WO 00 58466 A (MICANOVIC RADMILA ;LILLY CO ELI (US); RATHNACHALAM RADHAKRISHNAN () 5 October 2000 (2000-10-05) cited in the application page 13, paragraph 10; examples 1-9 page 24, paragraph 4 -page 26, paragraph 2	1-20

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
10 September 2002	20/09/2002

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Gurdjian, D

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	KRONMAN C ET AL: "Involvement of oligomerization, N-glycosylation and sialylation in the clearance of cholinesterases from the circulation." THE BIOCHEMICAL JOURNAL. ENGLAND 1 NOV 1995, vol. 311 (Pt 3), 1 November 1995 (1995-11-01), pages 959-967, XP008007650 ISSN: 0264-6021 abstract	1-20
A	PITTI ET AL: "Genomic amplification of a decoy receptor for FAS ligand in lung and colon cancer" NATURE, MACMILLAN JOURNALS LTD. LONDON, GB, vol. 396, 17 December 1998 (1998-12-17), pages 699-703, XP002139977 ISSN: 0028-0836 abstract; figure 1	1-20
A	YU K-Y ET AL: "A newly identified member of tumor necrosis factor receptor superfamily (TR6) suppresses LIGHT-mediated apoptosis" JOURNAL OF BIOLOGICAL CHEMISTRY, AMERICAN SOCIETY OF BIOLOGICAL CHEMISTS, BALTIMORE, MD, US, vol. 274, no. 20, 14 May 1999 (1999-05-14), pages 13733-13736, XP002161940 ISSN: 0021-9258 figure 1	1-20
A	BAI C ET AL: "OVEREXPRESSION OF M68/DCR3 IN HUMAN GASTROINTESTINAL TRACT TUMORS INDEPENDENT OF GENE AMPLIFICATION AND ITS LOCATION IN A FOUR-GENE CLUSTER" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, NATIONAL ACADEMY OF SCIENCE. WASHINGTON, US, vol. 97, no. 3, 1 February 2000 (2000-02-01), pages 1230-1235, XP002938755 ISSN: 0027-8424 abstract; figure 6	1-20
P,X	WO 01 18055 A (TIAN YU ;LILLY CO ELI (US); ATKINSON PAUL ROBERT (US); WITCHER DER) 15 March 2001 (2001-03-15) page 11, paragraph 2 -page 12, line 32	1-20

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 02/00509

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 1-20
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
see FURTHER INFORMATION sheet PCT/ISA/210

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 1-20

Present claims 1-20 relate to a compound defined (inter alia) by reference to the following parameter : 'said composition having an average sialic acid content of about ... sialic acids per molecule of FLINT analog ' .
The use of these parameter in the present context is considered to lead to a lack of clarity within the meaning of Article 6 PCT. It is impossible to compare the parameter the applicant has chosen to employ with what is set out in the prior art. The lack of clarity is such as to render a meaningful complete search impossible. Consequently, the search has been restricted to FLINT analogs and the general concept of sialylation .

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 02/00509

Patent document cited in search report		Publication date	Patent family member(s)		Publication date
WO 0058465	A	05-10-2000	AU	3739400 A	16-10-2000
			AU	3739500 A	16-10-2000
			EP	1165780 A2	02-01-2002
			EP	1165781 A2	02-01-2002
			WO	0058465 A2	05-10-2000
			WO	0058466 A2	05-10-2000
WO 0058466	A	05-10-2000	AU	3739400 A	16-10-2000
			AU	3739500 A	16-10-2000
			EP	1165780 A2	02-01-2002
			EP	1165781 A2	02-01-2002
			WO	0058465 A2	05-10-2000
			WO	0058466 A2	05-10-2000
WO 0118055	A	15-03-2001	AU	6891800 A	10-04-2001
			WO	0118055 A1	15-03-2001